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Author(s)	He, Weiyong; Ohashi, Kazuhiko; Sugimoto, Chihiro; Tsuji, Masayoshi; Onuma, Misao
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## Cloning and characterization of cDNA encoding a prohibitin-like protein from *Theileria orientalis*

Weiyong He<sup>1,2)</sup>, Kazuhiko Ohashi<sup>1)</sup>, Chihiro Sugimoto<sup>1,3)</sup>, Masayoshi Tsuji<sup>4)</sup>  
and Misao Onuma<sup>1)</sup>

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### Abstract

A cDNA clone encoding a prohibitin-like protein (Toprh) was isolated from a piroplasm cDNA library of *Theileria orientalis* and its nucleotide sequence was determined. An open reading frame, encoding a polypeptide of 278 amino acid residues, was found in *Toprh* cDNA sequence. An intron of 89 bp was identified when this cDNA clone was compared with the *Toprh* gene in the genome of *T. orientalis*. The deduced amino acid sequence of *Toprh* shares 93.8, 93.1 and 69.1% identities with the prohibitins of *T. parva* (from chromosome 1), *T. annulata* (from chromosome 1), and *Plasmodium falciparum*, (from chromosome 10), respectively. By Western blot analysis, Toprh was found to be expressed in the piroplasm stage of the parasites.

Key Words : *Theileria orientalis*, prohibitin, piroplasm

### Introduction

*Theileria* parasite is a tick-transmitted protozoan parasite of cattle, belonging to unicellular organisms. There are several species of *Theileria* recorded in cattle including *T. annulata*, *T. parva* and *T. orientalis*<sup>9)</sup>. *T. annulata* and *T. parva* are virulent while *T. orien-*

*talis* is a conditional pathogenic parasite, though it has been occasionally reported to cause economic losses in grazing cattle in Japan<sup>9)</sup>. Calves infected with *T. orientalis* suffer chronic anemia due to intraerythrocytic piroplasms with fluctuating parasitemia over time, and they occasionally die. Little is known about the mechanism of persistent in-

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<sup>1)</sup>Laboratory of Infectious Diseases, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, 060-0818, <sup>2)</sup>College of Veterinary Medicine, China Agricultural University, Beijing 100094, China, <sup>3)</sup>National Research Center for Protozoan Diseases, Obihiro University, Obihiro 080-8555 and <sup>4)</sup>Faculty of Veterinary Medicine, Rakuno Gakuen University, Ebetsu 069-8501, Japan  
Tel : 011-706-5215  
Fax : 011-706-5217  
E-mail : monuma@vetmed.hokudai.ac.jp

fection by *T. orientalis* and whether there is a relationship between the persistent infection and the programmed cell death.

Apoptosis (programmed cell death) is an important physiological process involved in development and homeostasis of multicellular and unicellular organisms. It has been also reported that apoptosis occurred in some protozoan parasites, including *Leishmania* spp.<sup>18)</sup>, *Plasmodium* spp.<sup>3)</sup>, and *Trypanosoma* spp.<sup>1)</sup>, though they may undergo apoptosis using apoptotic machinery that differs significantly from that of their host<sup>3)</sup>. Thus, characterization of the apoptotic process in these parasites could provide information regarding their pathogenesis, which could be exploited to target new drugs to limit their growth and treat the disease they cause<sup>3)</sup>. There are a series of proteins associated with the whole process of apoptosis. Among them, prohibitin is a group of highly conserved proteins which are thought to control the cell cycle, senescence, and tumor suppression<sup>7,11)</sup>. Prohibitin and the prohibitin-like protein BAP 37 have been localized to the plasma membrane of mouse lymphocytes, where they together interact with the IgM antigen receptor and may function in signaling apoptotic programmed cell death<sup>15)</sup>. Androgens target prohibitin to regulate proliferation of prostate cancer cells, which showed that regulation of growth-relation protein would be an ideal second line of therapy for cancers<sup>4,6,14)</sup>. Although the prohibitin gene has been identified in several intracellular protozoan parasite species, including *T. parva* and *T. annulata* (data from GenBank), no detailed characterization of the prohibitin gene and its gene product has been reported. In order to understand the molecular mechanism for apoptosis in *T. orientalis*, and to develop a new treatment method for theileriosis, characterization of the prohibitin-like gene of *T. orientalis*, whose gene product

could potentially be involved in apoptosis, was done in this study.

## Materials and Methods

### *Parasites*

Preparation and purification of *T. orientalis* was done as previously described<sup>12)</sup>. Briefly, a splenectomised calf was challenged with the cloned C type of *T. orientalis*<sup>16)</sup>. When the calf developed appreciable parasitemia, anticoagulant blood was collected, and white blood cells were removed by the filtration, while the infected erythrocytes were lysed with liquid nitrogen at the pressure of 1,000 psi for 2 min. *T. orientalis* were then extracted from the lysate by differential centrifugation.

### *Preparation of cDNA library of T. orientalis*

Isolation of total RNA from *T. orientalis* was done using the TRIzol reagent (GIBCO-BRL) following the instructions from the manufacturer. The cDNA library of the parasite was made using the SMART cDNA library construction kit (CLONTECH Laboratories Inc. USA) according to the instructions from the manufacturer. Briefly, to 1 mg of Total RNA, the primer (CDIII/ 3' PCR Primer) and the SMART III Oligonucleotide were added and the mixture was used to run the first-strand cDNA synthesis at 42° C for 1 hour. cDNA was amplified by LD PCR (5 sec at 95° C for initial denaturation, then 20 cycles of 5 sec at 95° C and 6 min at 68° C) using the primers in the kit. The product was treated with proteinase K at 45° C for 20 min, then purified and digested with *Sfi*I. The digested products were size-fractionated using CHROMA SPIN-400 (CLONTECH Laboratories, Inc.). The fractionated ds-cDNA fragments were ligated into the pDNR-LIB donor vector at 16° C overnight, and the recombinant vector was transfected into *E.coli* DH 5 $\alpha$ .

*DNA sequencing analysis of prohibitin-like protein gene and comparison with the genomic DNA of T. orientalis*

Random DNA sequencing analysis of plasmids prepared from colonies containing the cDNA library of the parasite was carried out to search target genes by using the sequencing primers in the SMART cDNA library construction kit (CLONTECH Laboratories Inc. USA), the Dye Terminator cycle sequencing system (Applied Biosystems, USA), and automated sequencers (310 Genetic Analyzer, Applied Biosystems). The template for sequencing was generated by PCR amplification of the cloned cDNA using the specific primers (1 min at 94°C for initial denaturation, 25 cycles of 30 sec. at 94°C, 30 sec at 55°C, and 2 min at 72°C, and then 7 min at 72°C for the final extension). Sequence analysis was done using the Genetyx-Mac software package (ver. 11, Software Development, Japan) as well as the GenBank and Swissprot databases for the comparison with known sequences. Alignment of the sequences and phylogenetic analyses using the neighbor-joining method were performed by the Clustal W program, provided by the DNA Data Bank of Japan (<http://www.ddbj.nig.ac.jp/Welcome-j.html>). Bootstrap support was assessed using 1,000 replicates. The nucleotide sequence of the Toprh cDNA has been submitted to GenBank and assigned accession number AB 161472.

After the determination of the sequence of the *Toprh* cDNA, the genomic DNA sequence of the *Toprh* gene was analyzed by using several new primers designed to amplify 200- to 300-bp subfragments from the *Toprh* gene.

*Southern blot analysis*

*T. orientalis* and bovine genomic DNA samples were digested with restriction en-

zymes, electrophoresed on a 1.1% agarose gel and blotted on to Hybond N<sup>+</sup> membrane (Amersham, UK) by the capillary transfer method<sup>12)</sup>. The probe was prepared by cutting the full-length Toprh cDNA plasmid with restriction enzymes, *EcoRV* and *Bcl I*, and purified using GeneClean kit (Bio. 101, USA). The final concentration of the probe fragment was adjusted to 200ng/14ml, and labeled with  $\alpha$ -<sup>32</sup>P dCTP using the multiprime DNA labeling kit (Amersham, UK) according to the instructions from the manufacture. Hybridization was carried out at 42°C overnight in a buffer containing 0.5M phosphate (pH 7.2), 1 mM EDTA and 0.5% SDS, and the membranes were routinely washed to a final stringency of 2 xSSC, 0.1% SDS at 65°C for 30min and subjected to autoradiography.

*Expression and purification of a truncated form of recombinant Toprh (tToprh) and preparation of antisera against tToprh*

A truncated form of recombinant prohibitin (tToprh) was expressed using the pET32 expression system. Briefly, a part (nt215-853, 213 amino acid residues) of the predicted coding sequence for the mature protein form of Toprh was expressed as a truncated fusion protein (tToprh) in an *E. coli* strain, AD494 DE 3 pLysS (Novagen, USA), by using the expression vector pET-32(a+), which produces a recombinant protein fused with histidine-tagged thioredoxin protein (TRX). The tToprh and the control protein, the 20kDa histidine-tagged TRX, was produced from the expression vector following the induction with IPTG, and purified by Ni-affinity chromatography according to the instructions from the manufacturer (Novagen, USA).

Antisera against tToprh were raised in 8-week-old Balb/c female mice. The immunogen was prepared by mixing 50µg of purified tToprh with 20µl of Gerbu Adjuvant. Three

mice were subcutaneously injected with the immunogen and boosted with the same one at the 3 rd, 5 th, 7 th and 8 th week, and serum was collected and used as antibody against Toprh.

#### *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting*

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the modified procedure<sup>19)</sup>. Briefly, the samples of purified piroplasm were treated at 100°C for 2 min with 0.0625M Tris-HCl (pH6.8) containing 2 % SDS and 5 % 2-mercaptoethanol, and separated on a 10% polyacrylamide gel, and transferred to the PVDF membrane (Amersham International, Amersham UK). The membrane was blocked with 5 % skimmed milk and probed with anti-Toprh serum and peroxidase-conjugated goat anti-mouse IgG diluted at a ratio of 1 : 1,000. Positive signals were detected with 3,3'-diaminobenzidine tetrahydrochloride and cobalt chloride.

## Results

#### *Isolation and sequence analysis of the Toprh cDNA from T. orientalis*

During the investigation of genes expressed in the piroplasm of *T. orientalis* by direct sequencing analysis of the cDNA library of *T. orientalis*, a cDNA clone of 896bp, coding Toprh, was identified. This cDNA has an open reading frame which could encode a polypeptide of 278 amino acid residues with a predicted molecular weight of 31.015kDa (Fig. 1 A). Sequence analysis of the *Toprh* gene in the parasite genome revealed that a 89-bp intron was present which was spliced out at the positions n128-129 of the *Toprh* cDNA (Fig. 1 A, 1B). Comparison of this deduced amino acid sequence with sequences in the DDBJ database showed that Toprh would belong to

the prohibitin family (Fig. 1C), and that the deduced amino acid sequence of Toprh showed 93.8 and 93.1% identities with those of prohibitins of *T. parva* (chromosome 1) and *T. annulata* (chromosome 1), respectively, and 69.1% identity with prohibitin of *Plasmodium falciparum* (chromosome 10). On the other hand, much lower identities were observed between Toprh and either prohibitins of *T. parva* (chromosome 3, 47.5%), *T. annulata* (chromosome 3, 46.9%), or *P. falciparum* (chromosome 8, 47.7%).

Phylogenetic analysis also showed that Toprh is more closely related to prohibitins of *T. parva* (chromosome 1), *T. annulata* (chromosome 1), and *P. falciparum* (chromosome 10) than to those of other species (Fig. 2). In addition, the tree illustrates that prohibitins of *T. parva* (chromosome 3) and *T. annulata* (chromosome 3), and *P. falciparum* (chromosome 8) into a separate cluster away from Toprh.

#### *Southern blot analysis*

In order to determine whether the *Toprh* gene originates from *T. orientalis*, Southern blot analysis was carried out by using bovine genomic DNA as a control (Fig. 3). The probe used hybridized only with the genomic DNA sample from purified *T. orientalis*, indicating that the *Toprh* gene is parasite-origin.

#### *Expression of Toprh in the piroplasm*

To examine the expression of the Toprh protein in *T. orientalis*, anti-serum was prepared against a truncated form of recombinant Toprh fused with the 20kDa histidine-tagged TRX (tToprh, 44kDa), and Western blot analysis was performed. Indeed, the anti-tToprh antiserum reacted specifically with a 44kDa band of tToprh (Fig. 4, lane 1). This antiserum also reacted with a band of about 33kDa, which is consistent with the molecular



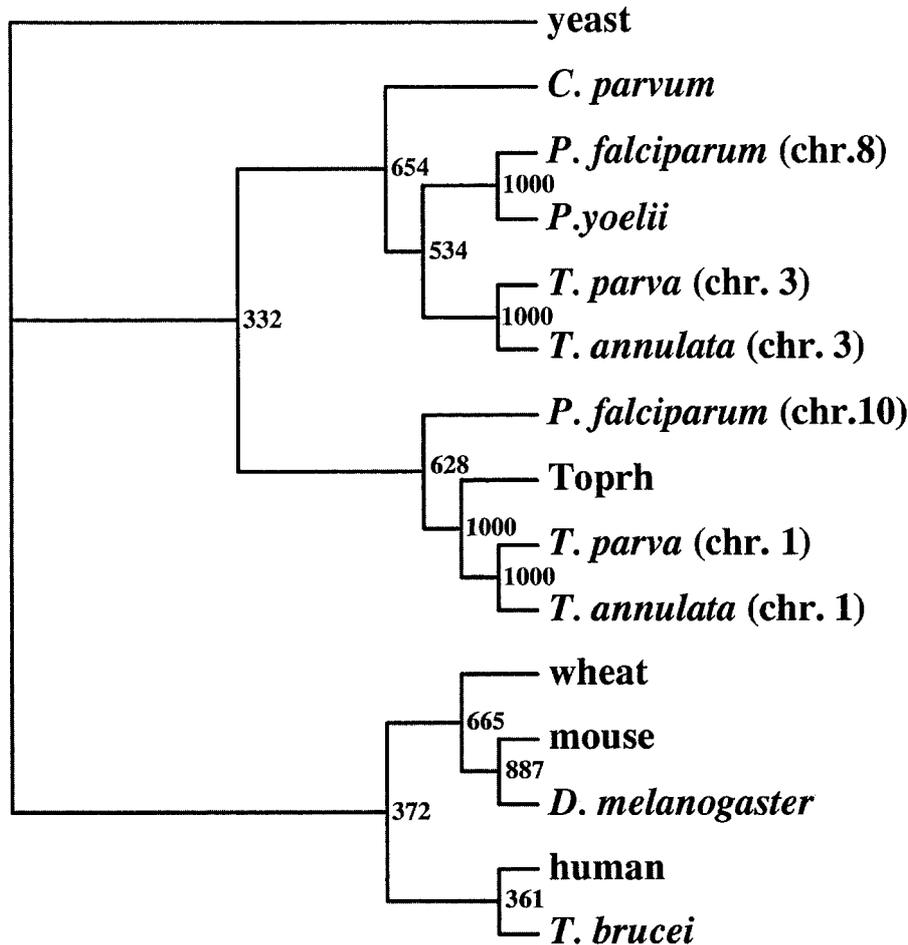


Fig. 2 . Phylogenetic tree analysis of prohibitin genes from several species. In addition to *Toprh* (accession number AB161472) , 12 nucleotide sequences of prohibitin genes from *T. parva* (AAGK 01000001, AAGK 01000005), *T. annulata* (CR 940347, CR 940352), *Trypanosome brucei* (AF 049901), *Plasmodium falciparum* (AL 844507, NC\_004314), *P. yoelii* (AABL 01002563), *Cryptosporidium parvum* (AAEE 01000001), wheat (AY 093954), yeast (V 16737), *Drosophila melanogaster* (NM\_165281), mouse (NM\_008831) and human (NM\_007273) were collected and used to construct the tree.

weight predicted from the deduced amino acid sequence of *Toprh*, when the extract from the purified piroplasm of *T. orientalis* was analyzed (Fig. 4, Lane 3). No specific reaction was observed in the lane of the extract from normal bovine RBC. These results suggest that *Toprh* was expressed in the stage of piroplasm.

## Discussion

The shape and wall structure of *T. orientalis* are changeable during the different stages of its life cycle, hampering us from using the usual unitary-vaccination way to effectively control the bovine theileriosis. Therefore, a novel alternative way would be necessary to prevent this disease, and knowledge on the molecular mechanisms of proliferation

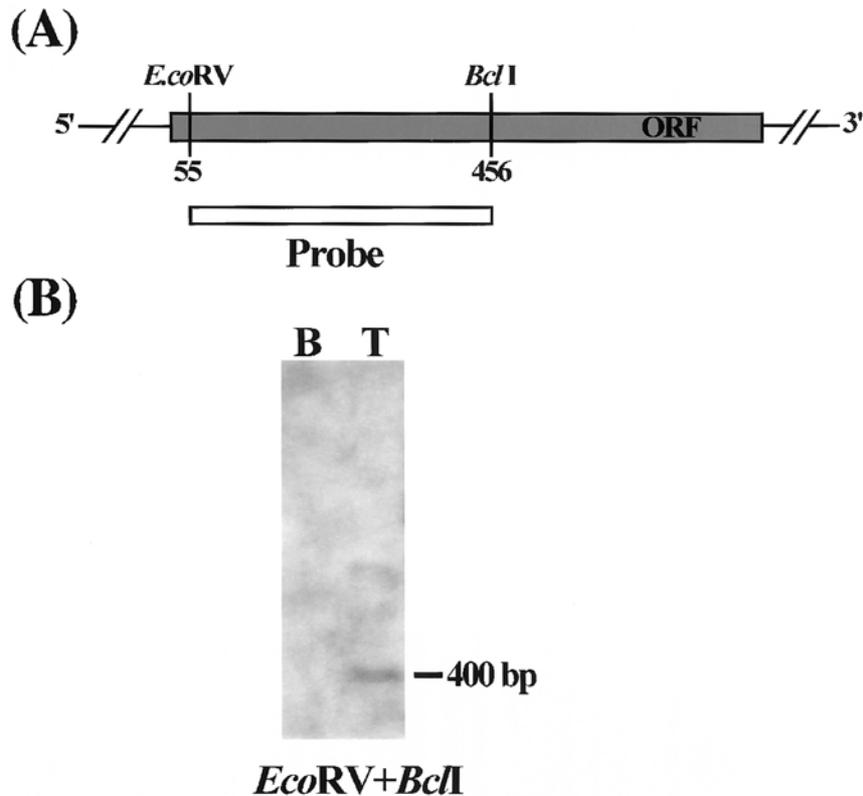


Fig. 3. Southern blot analysis of the *Toprh* gene. (A) The restriction enzyme map of the *Toprh* ORF, and the locations of the probe used. (B) The genomic DNA samples from *T. orientalis* (lane T) and cattle (lane B) as a negative control were digested with *BclI* and *EcoRV*, and hybridized with the probe.

and apoptosis of *T. orientalis* could help us to find a new supplementary method to control this parasites. Among the proteins related to regulation of cell proliferation, prohibitin, originally described as the product of a proto-oncogene in mammals, and its several biological functions involved in cell-cycle control and senescence were investigated<sup>2,7,8</sup>. For examples, prohibitin mutants of yeast resulted in the shortened replicative life span of yeast due to defective mitochondrial segregation in old mother cells<sup>10</sup>. A coiled-coil domain of prohibitin can repress E2F1-mediated transcription and induce apoptosis<sup>5</sup>. These observations suggest that the life of organism could be adjusted by the operation of prohibitin. It has been also reported that prohibitin, in ad-

dition to receptors for activated protein kinase C (RACK), is up-regulated in the terminally differentiated bloodstream form of *T. brucei rhodesiense* when apoptosis of this trypanosomes is induced by a lectin, Concanavalin A<sup>17</sup>. Yet, biological significance of prohibitin on the apoptotic process of protozoan parasites remains to be established.

In this study, *Toprh*, the prohibitin-like gene of *T. orientalis*, was identified, and shown to have high homology with the prohibitin genes of *T. parva*, *T. annulata*, and *P. falciparum*, indicating that the prohibitin gene is one of highly conserved genes among hemoprotozoa. However, other prohibitin genes were also reported in the case of *T. parva*, *T. annulata*, and *P. falciparum* (data from Gen-

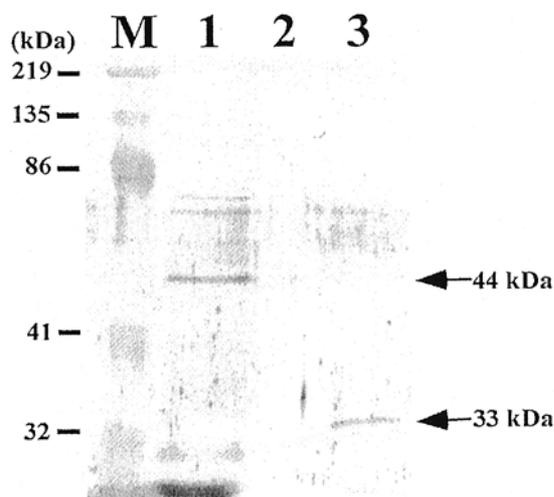


Fig. 4. Western blot analysis of Toprh. tToprh (lane 1), normal bovine RBC (lane 2), and purified *T. orientalis* (lane 3) were fractionated, blotted as described in the text, and probed with anti-tToprh antibody. Kaleidoscope prestained standards (Bio-Rad) was used as the molecular size makers, and the sizes were adjusted for the buffer system used in this experiment.

Bank), which form a cluster of the genes in the phylogenetic tree (Fig. 2). This result imply that another prohibitin gene may be present in the genome of *T. orientalis*. It will be necessary to determine whether another prohibitin gene is present in *T. orientalis* in the future. In addition, an intron was identified in the genomic DNA sequence of *Toprh*, which is the first gene containing introns found in the parasite of *T. orientalis*, suggesting that *T. orientalis* is a lower hemoprotozoan.

It was shown that the Toprh protein was detected in the *T. orientalis* piroplasm, indicating that Toprh was translated during the erythrocytic phase of the infection. Because the clinical signs of theileriosis is associated with the erythrocytic phase of the *T. orientalis* infection, the disease progression of theileriosis could be prevented by regulating the proliferation of piroplasm by means of apoptosis-associated molecules including prohibitin.

Further investigations are necessary to understand the role of prohibitin-like protein Toprh on the life cycle of *T. orientalis*.

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